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(54) Title: NUCLEAR-ENCODED TRANSCRIPTION SYSTEM IN PLASTIDS OF HIGHER PLANTS

(57) Abstract

The present invention provides novel DNA constructs and methods for stably transforming the plastids of higher plants. The constructs described herein contain unique promoters that are transcribed by both nuclear encoded plastid polymerases and plastid encoded plastid polymerases. Use of the novel constructs of the invention facilitates transformation of a wider range of plant species and enables tissue specific expression of a transforming DNA in plastids of multicellular plants.

NUCLEAR-ENCODED TRANSCRIPTION SYSTEM
IN PLASTIDS OF HIGHER PLANTS

FIELD OF THE INVENTION

The present invention relates to plant genetic engineering and particularly to plastid transformation in higher plants. The invention provides a novel promoter
5   sequences useful for the expression of foreign genes of interest in various plant species.

BACKGROUND OF THE INVENTION

10   Chloroplast genes are transcribed by an RNA polymerase containing plastid-encoded subunits homologous to the α, β and β' subunits of *E. coli* RNA polymerase. The promoters utilized by this enzyme are similar to *E. coli* σ^{70}-promoters, consisting of -35 and -10 consensus
15   elements (G.L. Igloi and H. Kossel, Crit. Rev. Plant Sci. 10, 525, 1992; W. Gruissem and J.C. Tonkyn, Crit. Rev. Plant. Sci. 12:-19, 1993) Promoter selection by the plastid-encoded RNA polymerase is dependent on nuclear-encoded sigma-like factors ((Link et al. 1994, *Plant*
20   *promoters and transcription factors*, Springer Verlag, Heidelberg, pp 63-83). In addition, transcription activity from some promoters is modulated by nuclear-encoded transcription factors interacting with elements upstream of the core promoter (L.A. Allison and P.
25   Maliga, *EMBO J.*, **14**:3721-3730; R. Iratni, L. Baeza, A. Andreeva, R. Mache, S. Lerbs-Mache, *Genes Dev.* **8**, 2928, 1994). These factors mediate nuclear control of plastid gene expression in response to developmental and environmental cues.
30   There has been speculation of the existence of a second transcription system in plastids. However, direct evidence to support such a speculation has

- 2 -

heretofore been unavailable. Identification of a novel
second transcription system in plastids represents a
significant advance in the art of plant genetic
engineering. Such a system enables greater flexibility
5   and range in plant species available for plastid
transformation, and facilitates tissue specific
expression of foreign proteins and RNAs via constructs
that can be manipulated by recombinant DNA techniques.

10   **SUMMARY OF THE INVENTION**
This invention provides DNA constructs and
methods for stably transforming plastids of multicellular
plants. The DNA constructs of the invention extend the
range of plant species that may be transformed and
15   facilitate tissue specific expression of foreign genes of
interest.
According to one aspect of the invention, DNA
constructs are provided that contain novel promoter
sequences recognized by a nuclear encoded plastid (NEP)
20   RNA polymerase. The DNA construct contains a
transforming DNA, which comprises a targeting segment,
at least one cloning site adapted for insertion of at
least one foreign gene of interest, the expression of the
foreign gene of interest being regulated by a promoter
25   recognized by a NEP polymerase, and a plastid selectable
marker gene.
The use of promoter elements recognized by
plastid encoded plastid (PEP) RNA polymerase for
enhancing expression of foreign genes of interest is
30   another aspect of the instant invention. Like the
constructs described above, these constructs also contain
a targeting segment, and a cloning site for expression of
a foreign gene of interest.
The promoters recognized by plastid encoded
35   plastid RNA polymerase have been well characterized in
photosynthetic tissues such as leaf. In contrast, the
nuclear-encoded polymerase transcription system of the

- 3 -

present invention directs expression of plastid genes
also in roots, seeds and meristematic tissue. In most
plants, including maize, cotton and wheat, plant
regeneration is accomplished through somatic

5   embryogenesis (i.e., involving meristematic tissue). In
a preferred embodiment of the invention, efficient
plastid transformation in these crops will be greatly
facilitated, through the use of the NEP plastid
transcription system, promoters and polymerases of the

10   present invention.

The NEP promoters of the invention are
incorporated into currently available plastid
transformation vectors and protocols for use thereof,
such as those described in U.S. Patent No. 5,451,513 and

15   pending U.S. Application No. 08/189,256, and also
described by Svab & Maliga., *Proc. Natl. Acad. Sci. USA*,
90, 913 (1993), the disclosures of which are all
incorporated herein by reference. To obtain transgenic
plants, plastids of non-photosynthetic tissues are

20   transformed with selectable marker genes expressed from
NEP promoters and transcribed by the nuclear-encoded
polymerase. Likewise, to express proteins of interest,
expression cassettes are constructed for high level
expression in non-photosynthetic tissue, using the NEP

25   promoter transcribed from the nuclear-encoded polymerase.
In another aspect of the invention, PEP promoters of the
invention are incorporated into currently available
plastid transformation vectors and protocols for use
thereof.

30   In yet another aspect of the invention, the NEP
transcription system also may be combined with the σ^{70}-
type system through the use of dual NEP/PEP promoters.

BRIEF DESCRIPTION OF THE DRAWINGS

35   **Fig. 1.** Deletion of *rpoB* from the tobacco
plastid genome by targeted gene replacement. (A)
Homologous recombination (diagonal lines) via plastid DNA

- 4 -

sequences flanking aadA in plasmid pLAA57 results in
replacement of rpoB (Sac I to Sma I fragment) in the
wild-type plastid genome (ptDNA) with aadA sequences,
yielding the ΔrpoB plastid genome (ΔrpoB ptDNA).

5   Abbreviations: rpoB, rpoC1, rpoC2 are plastid genes
encoding the β, β'and β'' subunits of the E.coli-like RNA
polymerase; aadA is a chimeric spectinomycin-resistance
gene. Restriction enzyme recognition sites: P, Pst I;
Sm, Sma I; Sc, Sac I. (B) Pigment deficiency is
10   associated with the deletion of rpoB. Total cellular DNA
was isolated from green (lanes 1,3,5) and white (lanes
2,4,6) leaf tissue from three independently transformed
lines (Line Nt-pLAA57-11B, lanes 1 and 2; line Nt-pLAA57-
16B, lanes 3 and 4; line Nt-pLAA57-18C, lanes 5 and 6)
15   and from wild-type green leaf tissue (Nt, lane 7). The
DNA was digested with Pst I, and the gel blot was
hybridized with a DNA fragment (nucleotide positions
22883-24486 of the ptDNA, numbering according to K.
Shinozaki, et al., (EMBO J. 5, 2043, 1986)) containing
20   part of rpoC1 (thick black line in Fig. 1A). The probe
hybridizes to a 9.0 kb fragment from the wild-type genome
and a 4.2 kb fragment from the ΔrpoB ptDNA. (C) DNA gel-
blot analysis confirms the lack of wild-type ptDNA copies
in white shoots of line Nt-pLAA57-10A (lane 2) and white
25   seed progeny of a grafted chimeric plant from the same
line (lane 3). DNA from wild-type green leaf tissue was
loaded in lane 1. Note the absence of the wild-type pt
DNA 9.0 kb fragment in ΔrpoB plants. The blot was
prepared as for Fig. 1B.

30

        Fig. 2. Deletion of rpoB results in a pigment-
deficient phenotype. (A) Green wild-type (left),
pigment-deficient ΔrpoB (right), and chimeric (center)
plants are shown. (B) A flowering chimeric plant in the
35   greenhouse. Note the white leaf margins indicating ΔrpoB
plastids in the second leaf layer which forms the
germline cells.

- 5 -

Fig. 3. (A) Plastids (P) in leaf mesophyll
cells of ΔrpoB plants lack organized photosynthetic
membranes. Abbreviations: N, nucleus; V, vesicles, M,
mitochondrion (B) For comparison an electron micrograph
5   of a wild-type leaf chloroplast (Cp) with thylakoid
membranes (T) is shown. Magnification in both (A) and
(B) is 7,800X.

Fig. 4. (Upper) Accumulation of plastid mRNAs
10   for (A) photosynthetic genes and (B) genetic system genes
in the ΔrpoB plants. Gel blots were prepared with total
cellular RNA (A, 3 μg per lane; B, 5 μg per lane) from
wild-type (lanes 1,3,5) and ΔrpoB (lanes 2,4,6) leaf
tissue, and hybridized to the indicated plastid gene
15   sequences. (Lower) Blots shown above were reprobed with
25S rDNA sequences. Hybridization signals were
quantified with a Molecular Dynamics PhosphorImager and
normalized to the 25S rRNA signal. The fold excess of
wild-type over ΔrpoB signal intensities for each probe is
20   shown below the lanes.

Fig. 5. Transcription in the ΔrpoB plants
initiates from a non-canonical promoter. (A) Primer
extension analysis was used to map the 5' ends of rbcL
25   and 16SrDNA transcripts in wild-type (lanes 1,3) and
ΔrpoB (lanes 2,4) plants. Primary transcripts are
marked by circles (open for wild-type, closed for ΔrpoB),
processed transcripts by a triangle. Transcripts of
unknown origin are starred. The accompanying sequence
30   ladders (loading order GATC) were generated using the
same primers that served in the primer extension
reactions. Numbers beside each extension product mark
the distance from the first nucleotide of the coding
sequence for rbcL and from the first nucleotide of the
35   mature 16S rRNA. (B) Mapping primary transcripts for 16S
rRNA in wild-type (lane 2) and ΔrpoB (lane 3) plants.
Total leaf RNA (20 μg) was capped in vitro, and capped

16S rRNA species were identified by RNAse protection
after hybridization with a complementary RNA probe.
Capped protected products are marked as in (A). Lane 1
contains RNA standards of the sizes indicated. (C) DNA

5   sequence of the *16SrDNA* upstream region with transcripts
initiating from promoters for the plastid-encoded (σ^{70}-
type, P1) and nuclear-encoded (P2) polymerases
(designation of P1 and P2 is based on A. Vera and M.
Sugiura, *Curr. Genet.* **27**, 280, 1995). Consensus σ^{70}

10 promoter elements (-35 and -10) are boxed. Initiation
sites are marked by circles, as in (A) and (B).
Numbering begins from the first nucleotide upstream of
the *16SrDNA* coding region (-1 = nucleotide 102757 in the
tobacco plastid genome).

15

        Fig. 6. Accumulation of plastid mRNAs in
wild-type and ΔrpoB tobacco leaves. Blots for the
plastid genes (see Example I) are grouped as follows. (A)
mRNA is significantly more abundant in the leaves of

20 wild-type than in ΔrpoB plants. (B) Levels of mRNA are
comparable in wild-type and ΔrpoB leaves, or (C) are
higher in ΔrpoB leaves. Gel blots were prepared with
total cellular RNA (3 µg per lane) from wild-type (lanes
1) and ΔrpoB (lanes 2) leaf tissue, and hybridized to

25 the indicated plastid gene sequences. (Lower panel) To
control for loading, blots shown above were reprobed with
25S rDNA sequences.

        Fig. 7. Mapping atpB transcription initiation

30 sites in wild-type and ΔrpoB tobacco leaves. (A) Primer
extension analysis. End-labeled primer extension
products from wild-type (wt) and ΔrpoB (T57) samples were
run alongside the homologous sequence obtained by using
the same primer. Numbers alongside the sequence refer to

35 the distance from the ATG translation initiation codon.
Primary transcripts from NEP and PEP promoters are marked
by filled and open circles, respectively. (B) In vitro

capping and RNase protection assay to identify primary
transcript 5' ends. Lanes were loaded with ΔrpoB (T57;
1, 2) and wild-type (wt; 4,5) RNA samples with (2,4) and
without (1,5) protecting complementary antisense RNA.
5   Molecular weight (MW) markers (100, 200, 300, 400, and
500 nucleotides) were loaded in lane 3. The transcript
5' end in (A) corresponds to the protected fragment size
in brackets: -254 (277nt), -289 (311). Note artifact
slightly below the 200 nt marker which is present in the
10 unprotected RNA samples. (C) Physical map of the atpB -
rbcL intergenic region. Map position of the primary
transcript 5' ends for the atpB NEP and PEP promoters are
marked as in (A).

15   **Fig. 8.** Mapping atpI transcription initiation
sites in wild-type and ΔrpoB tobacco leaves. (A) Primer
extension analysis. End-labeled primer extension
products from wild-type (wt) and ΔrpoB (T57) samples were
run alongside the homologous sequence obtained by using
20 the same primer. Numbers alongside the sequence refer to
the distance from the ATG translation initiation codon.
Primary transcripts from NEP and PEP promoters are
marked by filled and open circles, respectively. (B) In
vitro capping and RNAse protection assay to identify
25 primary transcript 5' ends. Lanes were loaded with ΔrpoB
(T57; 1, 2) and wild-type (wt; 4,5) RNA samples with
(2,4) and without (1,5) protecting complementary
antisense RNA. Molecular weight (MW) markers (100, 200,
300, 400, and 500 nucleotides) were loaded in lane 3. The
30 transcript 5' end in (A) corresponds to the protected
fragment size in brackets: -130 (235nt), -207, 209, 212
(303, 305, 309; not resolved). Note artifact slightly
below the 200 nt marker which is present in the
unprotected RNA samples. (C) Physical map of the rps2
35 -atpI intergenic region. Map position of the primary
transcript 5' ends for the atpI NEP and PEP promoters are
marked as in (A).

- 8 -

Fig. 9. Mapping clpP transcription initiation sites in wild-type and ΔrpoB tobacco leaves. (A) Primer extension analysis. End-labeled primer extension products from wild-type (wt) and ΔrpoB (T57) samples were run
5   alongside the homologous sequence obtained by using the same primer. Numbers alongside the sequence refer to the distance from the ATG translation initiation codon. Primary transcripts from NEP and PEP promoters are marked by filled and open circles, respectively. (B) In
10   vitro capping and RNAse protection assay to identify primary transcript 5' ends. Lanes were loaded with ΔrpoB (T57; 1, 2) and wild-type (wt; 4,5) RNA samples with (2,4) and without (1,5) protecting complementary antisense RNA. Molecular weight (MW) markers (100, 200,
15   300, 400, and 500 nucleotides) were loaded in lane 3. The transcript 5' end in (A) correspond to the protected fragment size in brackets: -53 (96 nt), -95 (138 nt), -173 (216 nt) and -511 (69 nt). Note artifact slightly below the 200 nt marker which is present in the
20   unprotected RNA samples. (C) Physical map of the clpP - psbB intergenic region. Map position of the primary transcript 5' ends for the clpP NEP and PEP promoters are marked as in (A).

25   Fig. 10. Mapping accD transcription initiation sites in wild-type and ΔrpoB tobacco leaves. (A) Primer extension analysis. End-labeled primer extension products from wild-type (wt) and ΔrpoB (T57) samples were run alongside the homologous sequence obtained by using
30   the same primer. Numbers alongside the sequence refer to the distance from the ATG translation initiation codon. Primary transcript for the PaccD-129 NEP promoter is marked by a filled circle. (B) In vitro capping and RNAse protection assay to identify primary transcript 5'
35   ends. Lanes were loaded with ΔrpoB (T57; 1, 2) and wild-type (wt; 4,5) RNA samples with (2,4) and without (1,5) protecting complementary antisense RNA. Molecular

weight (MW) markers (100, 200, 300, 400, and 500 nucleotides) were loaded in lane 3. The -57 transcript 5' end in (A) corresponds to the protected 103 nt fragment. Note artifact slightly below the 200 nt marker

5   which is present in the unprotected RNA samples. (C) Physical map of the accD -- rbcL intergenic region. Map position of the primary transcript 5' end for the PaccD-129 NEP promoter is marked.

10   **Fig. 11.** Alignment of DNA sequences flanking the NEP promoter transcription initiation sites. Nucleotides with more than 6 matches are boxed. Consensus sequence adjacent to the transcription initiation site is shown below. Position of 5' ends are marked by filled

15   circles. Note, that 5' ends for Prps12-152 and Prps16-107 were not capped and may not be primary transcripts.

          Fig. 12. NEP and PEP polymerases, through recognition of distinct promoters, provide a mechanism

20   for selective transcription of plastid genes. Note that some genes have only PEP promoters (photosystem I and photosystem II), others have both PEP and NEP promoters (most housekeeping genes), or only NEP promoters (accD).

25   **Fig. 13.** A schematic diagram of a chimeric plastid gene expressed from a NEP promoter.

DESCRIPTION OF THE INVENTION

          Several reports have suggested the existence of an additional plastid-localized, nuclear-encoded RNA

30   polymerase (reviewed in Gruissem and Tonkyn, 1993; Igloi and Kossel, 1992; Mullet, 1993; Link, 1994). By deleting the rpoB gene encoding the essential β subunit of the tobacco E. coli-like RNA polymerase. The existence of a second plastid transcription system which

35   is encoded by the nucleus has been established (Allison et al., 1996, EMBO J. **15**:2802-2809). Deletion of rpoB yielded photosynthetically defective, pigment-deficient

plants. An examination of plastid ultrastructure in leaf
mesophyll cells of the ΔrpoB plants revealed
proplastid-like organelles lacking the arrays of stacked
thylakoid membranes which are characteristic of

5   photosynthetically-active chloroplasts. Transcripts for
the rbcL, psbA and psbD photosynthetic genes were low,
whereas mRNAs for the rpl16, atpI and 16SrDNA genes
accumulated to about wild-type, or higher than in
wild-type levels. Lack of transcript accumulation for

10   the photosynthetic genes was due to lack of σ^{70}-type
promoter activity. While in wild-type tobacco leaves the
ribosomal RNA operon is normally transcribed from a
σ^{70}-type promoter, in the ΔrpoB plants the rRNA operon was
transcribed from a non-σ^{70} promoter. The rRNA operon is

15   the first transcription unit for which both a
plastid-encoded and nuclear-encoded plastid RNA
polymerase (PEP and NEP, respectively) was identified.
An analysis of the promoter regions of other
genes has revealed that the rRNA operon is not unique. It

20   is a member of a large class of plastid genes which have
at least one promoter each for PEP and NEP, with a
potential for expression by either of the two plastid RNA
polymerases. In addition, plastid genes have been
identified which are transcribed exclusively by NEP.

25   Furthermore, the data suggest that additional
gene-specific mechanisms regulate NEP transcript levels
in different plastid types.
A NEP transcriptional start site has been
identified about 62 bases upstream of the mature 16S rRNA

30   5' terminus. The sequence surrounding the initiation
site is highly conserved among numerous plant species
examined, and bears no resemblance to the PEP promoter
consensus sequence. NEP promoter consensus sequences
important for nuclear encoded polymerase recognition and

35   binding (analogous to the -10 and -35 sequences of the *E.
coli*-type transcription initiation site) are
preferentially located within about 50 nucleotides in

either direction of the NEP transcription start site. As described in greater detail in Example 1, several different NEP promoters exist, and NEP promoters are sometimes found in conjunction with PEP promoters.

5   The polymerases of the invention may be purified by chromatography, using standard methods. The NEP polymerase activity in column fractions can be assayed utilizing DNA segments comprising the NEP promoter region as templates in *vitro* transcription

10   reactions. Alternatively, NEP promoter segments may be attached to a matrix which is separable by some means (e.g., magnetic beads). The matrix-bound DNA is incubated with a plant extract under conditions in which the nuclear encoded polymerase is expected to bind the

15   DNA. The matrix/DNA/polymerase complex is then separated from the plant extract, and the bound protein may then be isolated and characterized. The protein purified by either of the above mentioned protocols may be used to produce antibodies to probe expression libraries, for the

20   purpose of isolating the nuclear genes or cDNAs encoding the nuclear encoded polymerase.

          As an alternative approach for the isolation of the NEP polymerase, proteins with specific affinity for the promoter fragment can be isolated and the N-terminal

25   amino acid sequence can be determined by microsequencing. The amino acid sequence can then be used to design appropriate PCR primers for gene isolation.

          The activity of the previously-known plastid-encoded σ^{70}-type transcription system has been well

30   characterized in photosynthetically active tissues, such as leaf. In contrast, the nuclear-encoded polymerase transcription system of the present invention directs expression of plastid genes also in roots, seeds and meristematic tissue. In most plants, including maize,

35   cotton and wheat, plant regeneration is accomplished through somatic embryogenesis (i.e., involving meristematic tissue). Efficient plastid transformation

- 12 -

in these crops will be enabled, or greatly facilitated, through the use of the NEP plastid transcription system of the present invention.

The NEP promoters of the invention can be
5   incorporated into currently available plastid transformation vectors and protocols for use thereof, such as those described in U.S. Patent No. 5,451,513 and pending U.S. Application No. 08/189,256, and also described by Svab & Maliga., *Proc. Natl. Acad. Sci. USA*,
10   90, 913 (1993), all of which are incorporated herein by reference. To obtain transgenic plants, plastids of non-photosynthetic tissues are transformed with selectable marker genes expressed from NEP promoters and transcribed by the nuclear-encoded polymerase. Likewise, to express
15   proteins of interest, expression cassettes are constructed for high level expression in non-photosynthetic tissue, using the NEP promoter transcribed from the nuclear-encoded polymerase. The NEP transcription system also may be combined with the σ^{70}-
20   type system through the use of dual NEP/PEP promoters. In some cases, expression of transgenes from NEP promoters in photosynthetic tissue also may be desirable.

The detailed description set forth in Examples I-III below describes preferred methods for making and
25   using the DNA constructs of the present invention and for practicing the methods of the invention. Any molecular cloning and recombinant DNA techniques not specifically described are carried out by standard methods, as generally set forth, for example, in Ausubel (ed.),
30   Current Protocols in Molecular Biology. John Wiley & Sons, Inc.(1994).

The following nonlimiting Examples describe the invention in greater detail.

35   **EXAMPLE 1**
                Demonstration of a Second Distinct Plastid
                Transcription System by Deletion of *rpoB*

To establish the existence of a non-*E. coli*-

like RNA polymerase in plastids, the gene for one of the essential subunits of the *E. coli*-like enzyme was deleted from the tobacco plastid genome. mRNA levels were then assessed in mutant plastids. The data indicate that, in

5   the absence of the plastid-encoded *E. coli*-like enzyme, expression of some photogenes is dramatically reduced. In contrast, transcript levels for the plastid genes encoding the gene expression apparatus are similar to levels in wild-type plants. Therefore the non-*E.coli*-

10   like RNA polymerase selectively transcribes a subset of plastid genes. This second transcription apparatus does not initiate from typical *E. coli* σ^{70}-promoters but recognizes a novel promoter sequence.

15

Materials and Methods for Example I

Plasmid construction. Plasmid pLAA57 is a pBSKS+ (Stratagene) derivative which carries a Sac I to Bam HI fragment (nucleotides 22658 to 29820) of the

20   ptDNA. An internal Sac I to Sma I DNA fragment within the ptDNA insert, between nucleotides 24456 and 28192, was replaced by a chimeric spectinomycin-resistance (*aadA*) gene. The *aadA* gene is identical to that described (Z. Svab and P. Maliga, *Proc. Natl. Acad. Sci.*

25   *USA,* 90, 913, 1993), except that the *psbA* 3' region is shorter and is contained in an Xba I to Dra I fragment as described (J.M. Staub and P. Maliga, *Plant J.* 6, 547, 1994).

30   *Plant Transformation.* For plastid transformation tungsten particles were coated (Z. Svab and P. Maliga, *Proc. Natl. Acad. Sci. USA,* 90, 913, 1993) with pLAA57 DNA, and introduced into the leaves of *Nicotiana tabacum* plants using the DuPont PDS1000He

35   Biolistic gun at 1100 p.s.i. Transgenic shoots were selected aseptically on RMOP medium (Z. Svab, P. Hajdukiewicz, P. Maliga, *Proc. Natl. Acad. Sci. USA* 87,

- 14 -

8526, 1990) containing 500mg/ml spectinomycin
dihydrochloride. Transgenic cuttings were rooted and
maintained on RM medium consisting of agar-solidified MS
salts (T. Murashige and F. Skoog, *Physiol. Plant.*, 15,
5   493, 1962) containing 3% sucrose.

          Electron Microscopy. Electron microscopy was
done on fully expanded leaves from wild-type and ΔrpoB
cuttings grown in sterile culture on RM medium with 3%
10   sucrose. Tissue was fixed for 2 hours in 2%
glutaraldehyde, 0.2M sucrose, 0.1M phosphate buffer (pH
6.8) at room temperature, and washed three times in 0.2M
sucrose, 0.1M phosphate buffer. Fixed tissues were
postfixed in buffered 1% osmium tetroxide with 0.2M
15   sucrose, dehydrated in a graded ethanol series, embedded
in Spurr's epoxy resin (hard), sectioned, and stained
with uranyl acetate and lead citrate for transmission
electron microscopy.

20   *Gel blots*. Total leaf DNA was prepared as
described (I.J. Mettler, *Plant Mol. Biol. Rep.*, 5, 346,
1987), digested with restriction endonuclease Pst I,
separated on 0.7% agarose gels, and transferred to Hybond
N (Amersham) using the Posiblot Transfer apparatus
25   (Stratagene). Hybridization to a random-prime labeled
fragment was carried out in Rapid Hybridization Buffer
(Amersham) overnight at 65°C. Total leaf RNA was prepared
using TRIzol (GIBCO BRL), following the manufacturer's
protocol. The RNA was electrophoresed on 1%
30   agarose/formaldehyde gels, then transferred to nylon
membrane and probed as for the DNA blots.

          Synthesis of Probes. Double-stranded DNA
probes for *psbA*, *atpI*, and *rpl16* were prepared by random-
35   primed ^{32}P-labeling of PCR-generated DNA fragments. The
sequence of the primers used for PCR, along with their
positions within the tobacco ptDNA (K. Shinozaki, *et al.*

- 15 -

, *EMBO J*. 5, 2043, 1986) are as follows: *psbA*, 5' primer
= 5'-CGCTTCTGTAACTGG-3' (complementary to nucleotides
1550 to 1536 of the ptDNA), 3' primer = 5'-
TGACTGTCAACTACAG-3' (nucleotides 667 to 682); *atpI* 5'

5   primer = 5'-GTTCCATCAATACTC-3' (complementary to
nucleotides 15985 to 15971), 3' primer = 5'-
GCCGCGGCTAAAGTT-3' (nucleotides 15292 to 15306); *rpl16* 5'
primer = 5'-TCCCACGTTCAAGGT-3' (complementary to
nucleotides 84244 to 84230), 3' primer = 5'-

10   TGAGTTCGTATAGGC-3' (nucleotides 83685 to 83699). To
generate probes for *rbcL*, *psbD/C* and 16S rRNA, the
following restricted DNA fragments were ^{32}P-labeled: *rbcL*,
a Bam HI fragment (nucleotides 58047 to 59285 in the
ptDNA); *psbD/C*, a Sac II to Hind III fragment of the

15   tobacco *psbD/C* operon (nucleotides 34691-36393); 16S
rRNA, an Eco RI to Eco RV fragment (nucleotides 138447 to
140855 in the ptDNA).

The probe for tobacco 25S rRNA was from plasmid
pKDR1 (D. Dempsey, K.W. Wobbe, D.F. Klessig, *Mol. Plant*

20   *Path*. 83, 1021, 1993) containing a 3.75 kb Eco RI
fragment from a tobacco 25S/18S locus cloned in plasmid
pBR325. When hybridizing gel-blots for 25S rRNA, ^{32}P-
labeled double-stranded DNA probe was mixed with
unlabeled plasmid pKDR1 corresponding to a 2-fold excess

25   over the amount of RNA present on the filter.

Normalizing DNA levels by plastid genome copy
number. To test whether changes in plastid genome copy
number contributed to the estimated differences in gene

30   expression, total cellular DNA and RNA were prepared from
equal amounts of leaf tissue from wild-type and Δ*rpoB*
plants. To compare the number of plastid genome copies
per equivalent leaf mass, DNA gel-blots were carried out
with an equal volume of each DNA preparation and probed

35   with a radiolabeled Eco RI to Eco RV fragment (from
nucleotides 138447 to 140845 of ptDNA (K. Shinozaki, *et*
al., *EMBO J*. 5, 2043, 1986) of *16SrDNA* sequence.

- 16 -

Quantitation by PhosphorImage analysis demonstrated an
equal number of plastid genome copies in each sample.
The amount of 16S rRNA from equal tissue samples, as
measured by RNA gel-blots on equal volumes of each RNA
5   preparation, was reduced by 2.5-fold in the ΔrpoB plants.
This value is similar to the 3-fold reduction estimated
when normalizing with the cytoplasmic 25S rRNA signal
(Fig. 3B).

10   *Primer extension reactions*. Primer extension
reactions were carried out on 3 μg (wild-type) or 10 μg
(ΔrpoB) of total leaf RNA as described (L.A. Allison and
P. Maliga, *EMBO J.*, in press) using the following
primers: 16S rRNA: 5'-TTCATAGTTGCATTACTTATAGCTTC-3'
15   (complementary to nucleotides 102757-102732); *rbcL*: 5'-
ACTTGCTTTAGTCTCTGTTTGTGGTGACAT (complementary to
nucleotides 57616-57587). Sequence ladders were
generated with the same primers using the Sequenase II
kit (USB).
20

              *Identification of primary transcripts by in
vitro capping*. Total leaf RNA (20 μg) from wild-type and
ΔrpoB plants was capped in the presence of [α-^{32}P]GTP
(J.C. Kennell and D.R. Pring, *Mol. Gen. Genet.* 216, 16,
25   1989). Labeled 16S rRNAs were detected by ribonuclease
protection (A. Vera and M. Sugiura, *Plant Mol. Biol.* 19,
309, 1992) using the RPAII kit (Ambion). To prepare the
protecting complementary RNA, the *16SrDNA* upstream region
(nucleotides 102526-102761 of the ptDNA) was PCR-
30   amplified using the following primers: 5' primer was 5'-
CCTCTAGA<u>CCCTAAGCCCAATGTG</u>-3' corresponding to nucleotides
102526 and 102541 of the ptDNA (K. Shinozaki, *et al.* ,
EMBO J. 5, 2043, 1986), underlined) plus an XbaI site; 3'
primer was 5'-CCGGTACC<u>GAGATTCATAGTTGCATTAC</u>-3'
35   complementary to nucleotides 102761 to 102742 of the
ptDNA (underlined) plus a Kpn I site. The amplified
product was cloned as an Xba I to Kpn I fragment into Xba

- 17 -

I and Kpn I-restricted pBSKS+ vector (Stratagene). To
generate unlabeled RNA complimentary to the 5' end of 16S
rRNAs, the resulting plasmid was linearized with Xba I,
and transcribed in a Megascript (Ambion) reaction with T3
5   RNA polymerase. Markers (100, 200, 300, 400, and 500
nucleotides) were prepared with the RNA Century Markers
Template Set (Ambion), following the manufacturer's
protocol. The 72 nucleotide marker was the mature
processed transcript from the plastid *trnV* gene, and was
10   generated by RNAse protection.

15

Results and Discussion

   Disruption of the E. coli-*like RNA polymerase
activity in tobacco plastids results in a pigment-*
20   *deficient phenotype.* To avoid disrupting plastid genes
for other functions the *rpoB* gene was targeted for
deletion, since it is the first reading frame of an
operon encoding exclusively subunits of the *E. coli*-like
plastid polymerase (K. Shinozaki, *et al.* , *EMBO J.* 5,
25   2043, 1986). The deletion was accomplished by replacing
most of the *rpoB* coding region (3015 out of 3212 base
pairs) and 691 bp of upstream non-coding sequence, with a
chimeric spectinomycin resistance (*aadA*) gene (Z. Svab
and P. Maliga, *Proc. Natl. Acad. Sci. USA,* **90**, 913, 1993)
30   in a cloned plastid DNA (ptDNA) fragment. The resulting
plasmid was introduced by particle bombardment into
tobacco chloroplasts, where the *aadA* gene integrated into
the plastid genome via flanking plastid DNA sequences as
diagrammed in Fig. 1A. Since the plastid genetic system
35   is highly polyploid, with every leaf cell containing up
to 10,000 identical copies of the ptDNA, selective
amplification of transformed genome copies was carried

- 18 -

out by growing the bombarded tissue on spectinomycin-
containing medium (P. Maliga, *Trends Biotechnol*. 11, 101,
1993).

5   From the initial round of selection several
spectinomycin-resistant plants exhibiting sectors of
white leaf tissue were obtained (Fig. 2A). DNA gel-blot
analysis of white and green sectors indicated that the
pigment-deficiency was correlated with deletion of *rpoB*
in three independently transformed lines (Fig. 1B). Most
10   DNA samples from the pigment-deficient tissue, for
example lane 4 in Fig. 1B, contained a mix of wild-type
and transformed genome copies. The complete absence of
wild-type ptDNA copies was critical for the
interpretation of the data. Therefore, to obtain plants
15   containing only transformed plastid genomes, shoots were
regenerated from the white tissue sectors. This
procedure yielded uniformly white plants (Fig. 2A) which
contained no wild-type ptDNA as judged by DNA gel-blot
analysis (Fig. 1C). Regeneration from these white leaves
20   on spectinomycin-free medium yielded exclusively pigment-
deficient shoots, confirming the complete absence of
wild-type plastid genomes in all leaf layers and cell
types.

        It is difficult to obtain seed from tobacco
25   plants grown in sterile culture. Fortuitously, during
plant regeneration from primary transformants, we
obtained a periclinal chimera (S. Poethig, *Trends
Genetics* 5, 273, 1989) homoplasmic for the plastid
mutation in the L2 leaf layer (Fig. 2A). This line was
30   grafted on wild-type tobacco and was raised to maturity
in the greenhouse (Fig. 2B). Seed from self-pollinated
flowers gave rise to uniformly white seedlings, in which
no wild-type plastid genomes could be detected by DNA
gel-blot analysis (Fig. 1C).

35

        *Plastids in leaves of the ΔrpoB plants lack
thylakoid membranes*. The pigment-deficient ΔrpoB plants

were unable to grow photoautotrophically. However, if
maintained on sucrose-containing medium to compensate for
their lack of photosynthesis, they grew normally but at a
reduced rate compared to wild-type plants, and exhibited

5   no noticeable changes in organ morphology. Moreover,
Δrpob seedlings germinated at a high efficiency, and
developed into plants. These observations indicate that
the *E. coli*-like plastid RNA polymerase is not required
for maintenance of the nonphotosynthetic plastid

10   functions necessary for plant growth and differentiation.

              An examination of plastid ultrastructure in
leaf mesophyll cells of the Δrpob plants revealed that
the mutant plastids were smaller and rounder than wild-
type chloroplasts, averaging 2-5 μm in length as compared

15   to 5-9 μm for wild-type chloroplasts. The Δrpob plastids
are thus larger than undifferentiated proplastids whose
average size is 1 μm (M.R. Thomas and R.J. Rose, *Planta*
158, 329, 1983). In addition, Δrpob plastids typically
contained multiple vesicles of irregular size and shape,

20   and lacked the arrays of stacked thylakoid membranes
which are characteristic of photosynthetically-active
chloroplasts (Fig. 3).

25   *Transcription of plastid genes is maintained in*
Δrpob *plastids*. In the absence of the β subunit, no
transcription was expected from plastid σ^{70}-type
promoters. To determine whether any transcription
activity was maintained in the Δrpob plastids,

30   accumulation of RNAs was examined by RNA gel-blot
analysis. Transcripts were surveyed for two different
classes of plastid genes (K. Shinozaki, *et al.*, *EMBO J.*
5, 2043, 1986). The first group included genes encoding
subunits of the photosynthetic apparatus: the *psbD/C*

35   operon, encoding subunits D2 and CP43 of photosystem II;
rbcL, encoding the large subunit of ribulose-1,5-

- 20 -

bisphosphate carboxylase; and *psbA*, encoding the D1
subunit of the photosystem II reaction center. The
second group contained genes for components of the gene
expression apparatus: *rpl16* encoding a ribosomal protein
5   subunit, and the *16SrDNA* gene. All plastid RNA
quantitations were normalized to cytoplasmic 25S
ribosomal RNA levels.

          Surprisingly, accumulation of mRNAs was
detected for all the genes examined. However, the effect
10   of the *rpoB* deletion on transcript accumulation was
dramatically different for the two classes of genes. The
steady-state mRNA levels of the photosynthetic genes
psbD/C, *rbcL*, and *psbA*, were reduced 40- to 100-fold
compared to wild-type levels (Fig. 4A; signals were
15   visible in all Δ*rpoB* lanes upon longer exposure). In
contrast, transcript levels for nuclear encoded
polymerase genes were much less affected. A 3-fold
reduction for 16S rRNA was measured, and an actual
increase for the multiple transcripts arising from the
20   polycistronic operon containing the *rpl16* gene was also
observed (Fig. 4B). These data indicate that, while
expression of genes encoding the photosynthetic apparatus
is defective in the Δ*rpoB* plants, the RNAs for genes
involved in housekeeping functions accumulate to
25   approximately wild-type, or higher, levels.

          The **16SrDNA** *gene is transcribed from a novel*
promoter in Δ*rpoB plants*. The accumulation of plastid
RNAs confirmed that there is RNA polymerase activity in
30   plastids lacking the β subunit of the *E. coli*-like
enzyme. However, migration of plastid genes to the
nucleus has been documented (S.L. Baldauf and J.D.
Palmer, *Nature* **334**, 262, 1990; J.S. Gantt, S.L. Baldauf,
P.J. Calie, N.F. Weeden, J.D. Palmer, *EMBO J.* **10**, 3073,
35   1991; M.W. Gray, *Curr. Op. Genet. Dev.* **3**, 884, 1993).
Therefore, transcription in Δ*rpoB* plastids could still

- 21 -

conceivably initiate from σ^{70}-type promoters if there existed a nuclear copy of *rpoB*, whose product could be imported into plastids and assembled into functional *E. coli*-like enzyme. To establish whether the plastid

5   transcripts detected in Δ*rpoB* plants were products of transcription from a σ^{70}-type promoter, the 5' transcript ends for four genes were mapped, *rbcL* (K. Shinozaki and M. Sugiura, *Gene 20*, 91, 1982), *16SrDNA* (A. Vera and M. Sugiura, *Curr. Genet.* 27, 280, 1995), *psbA* (M. Sugita and

10   M. Sugiura, *Mol. Gen. Genet.* 195, 308, 1984) and *psbD* (W.B. Yao, B.Y. Meng, M. Tanaka, M. Sugiura, *Nucl. Acids Res.* 17, 9583, 1989), for which the transcription initiation sites have been established previously. None of the 5' ends mapped to σ^{70}-type promoter initiation

15   sites (data are shown for *rbcL* and *16SrDNA* in Fig. 5A). Therefore it was concluded that the residual RNA polymerase activity in the Δ*rpoB* plastids was not due to an *E. coli*-like enzyme, but represents a second unique plastid transcription system. This distinct RNA

20   polymerase enzyme is referred to as the Nuclear Encoded Plastid RNA polymerase (NEP), to distinguish it from the *E. coli*-like enzyme which we designate Plastid Encoded Plastid RNA polymerase (PEP). Since the tobacco plastid genome has been fully sequenced, and since the few

25   unidentified reading frames bear no sequence similarity to known RNA polymerase subunits (K. Shinozaki, *et al.* , *EMBO J*. 5, 2043, 1986), transcription by the nuclear encoded RNA polymerase relies on nuclear gene products.
                    In the absence of transcription from σ^{70}-

30   type promoters in the Δ*rpoB* plants, the question remained: what promoters were the source of the plastid RNAs. The 16S rRNA 5' end detected in the Δ*rpoB* plants mapped 62 nucleotides upstream of the mature 16S rRNA 5' terminus (Fig. 5A). This 5' end was determined to be a

35   primary transcript by *in vitro* capping (Fig. 5B). A prominent primary transcript with a similar 5' end was recently reported in proplastids of heterotrophically-

- 22 -

cultured tobacco cells, and was designated P2 (A. Vera
and M. Sugiura, *Curr. Genet.* 27, 280, 1995; This
transcript is also present at very low levels in wild-
type leaf cells (A. Vera and M. Sugiura, *Curr. Genet.*
5   27, 280, 1995; Fig. 5A longer exposure, not shown). The
sequence surrounding the initiation site is highly
conserved among all plant species examined, and bears no
resemblance to the σ^{70} consensus sequence (A. Vera and M.
Sugiura, *Curr. Genet.* 27, 280, 1995). Based on its
10 prominent usage in the Δ*rpoB* plants, it was concluded
that this unique promoter is utilized by the NEP
transcription apparatus.

In contrast to the 16S rRNA, the major
transcripts for the photosynthetic genes *rbcL*, and *psbD/C*
15 mapped to previously-characterized processed ends (data
shown for *rbcL* Fig 5; L. Hanley-Bowdoin, E.M. Orozco, N.-
H. Chua, *Mol. Cell. Biol.* 5, 2733, 1985; J.E. Mullet,
E.M. Orozco, N.-H. Chua, *Plant Mol. Biol.* 4, 39, 1985; S.
Reinbothe, C. Reinbothe, C. Heintzen, C. Seidenbecher, B.
20 Parthier, *EMBO J.* 12, 1505, 1993). Additional minor
transcript ends mapped upstream of the processed termini.
Therefore, the low levels of transcript accumulation for
these photosynthetic genes are the result of upstream
promoter activity and subsequent processing of the
25 readthrough RNAs to yield correctly-sized transcripts.

*Proposed roles for the two plastid
transcription systems*. In the Δ*rpoB* plants there is
30 accumulation of RNAs transcribed by the NEP system. This
indicates a role for the nuclear encoded RNA polymerase
in maintaining the expression of plastid housekeeping
genes. Apparently these expression levels are sufficient
to support the growth and differentiation of non-
35 photoautotrophic plants. In contrast, the *E. coli*-like

- 23 -

PEP RNA polymerase is required to provide the high levels
of plastid gene transcripts necessary for development of
photosynthetically active chloroplasts. The proposed
role for the nuclear encoded RNA polymerase implies a
5   high demand for its function during the early phases of
chloroplast development, before the PEP RNA polymerase is
active (J.E. Mullet, *Plant Physiol.*, **103**, 309, 1993).
Developmental regulation of a nuclear-encoded RNA
polymerase is supported by the observation that the
10   nuclear encoded polymerase P2 promoter of the *16SrDNA*
gene is more active in proplastids of cultured tobacco
cells than in leaf chloroplasts (A. Vera and M. Sugiura,
Curr. Genet. **27**, 280, 1995).

15

EXAMPLE II
Transcription by two distinct RNA polymerases
is a general regulatory mechanism
of gene expression in higher plants

20

As described in Example I, accumulation of
transcripts in plants lacking the PEP polymerase led to
25   the identification of a NEP promoter for the plastid
ribosomal RNA operon (Allison et al. 1996, EMBO J.
14:3721-3730). To facilitate mapping of additional NEP
promoters, mRNA accumulation was examined in ΔrpoB plants
for most classes of plastid genes. The novel promoter
30   sequences described herein may be used to extend the
range of species within such plastid transformation is
feasible and to drive expression of foreign genes of
interest in a tissue specific manner.

35   **Materials and Methods for Example II**

RNA Gel blots Total leaf RNA was prepared
using TRIzol (GIBCO BRL), following the manufacturer's

- 24 -

protocol. The RNA was electrophoresed on 1%
agarose/formaldehyde gels, then transferred to Hybond N
(Amersham) using the Posiblot Transfer apparatus
(Stratagene). Hybridization to random-primer labeled

5   fragment was carried out in Rapid Hybridization Buffer
(Amersham) overnight at 65°C. Double-stranded DNA probes
were prepared by random-primed ^{32}P-labeling of
PCR-generated DNA fragments. The sequence of the primers
used for PCR, along with their positions within the

10   tobacco ptDNA (Shinozaki, et al. , 1986, supra) are as
follows:

Gene	5' nucleotide position in plastid DNA	Sequence
accD	60221	GGATTTAGGGGCGAA
	60875	GTGATTTTCTCTCCG
atpB	56370(C)	AGATCTGCGCCCGCC
	55623	CCTCACCAACGATCC
atpI	15985(C)	GTTCCATCAATACTC
	15292	GCCGCGGCTAAAGTT
clpP	73621(C)	GACTTTATCGAGAAAG
	73340	GAGGGAATGCTAGACG
ndhA	122115(C)	GATATAGTGGAAGCG
	121602	GTGAAAGAAGTTGGG
ndhB	97792(C)	CAGTCGTTGCTTTTC
	97057	CTATCCTGAGCAATT
ndhF	113366(C)	CTCGGCTTCTTCCTC
	112749	CTCCGTTTTTACCCC
ORF1901	129496(C)	GTGACTATCAAGAGG
	128895	GACTAACATACGCCCG
ORF2280	92881	GCTCGGGAGTTCCTC
	93552	TGCTCCCGGTTGTTC
petB	78221	GGTTCGAAGAACGTC
	78842	GGCCCAGAAATACCT
psaA	43467(C)	TTCGTTCGCCGGAACC
	42743	GATCTCGATTCAAGAT
psbB	75241	GGAGCACATATTGTG
	75905	GGATTATTGCCGATG
psbE	66772(C)	CAATATCAGCAATGCAGTTCATCC
	66452	GGAATCCTTCCAGTAGTATCGGCC
rps14	38621	CACGAAGTATGTGTCCGGATAGTCC
rpl33/rpl18	70133	GGAAAGATGTCCGAG
	70636	GTTCACTAATAAATCGAC

The rps16 mRNA was probed with an EcoRI
fragment isolated from plasmid pJS40, containing

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- 26 -

sequences between nucleotides 4938/5363 and 6149/6656 of
the tobacco ptDNA (Shinozaki et al., 1986, supra). The
probe for tobacco 25S rRNA was from plasmid pKDR1
(Dempsey et al., Mol. Plant Path. 83:1021, 1993)

5   containing a 3.75 kb EcoRI fragment from a tobacco
25S/18S locus cloned in plasmid pBR325. When hybridizing
gel-blots for 25S rRNA, ^{32}P-labeled double-stranded DNA
probe was mixed with unlabeled plasmid pKDR1
corresponding to a 2-fold excess over the amount of RNA

10   present on the filter.

Primer extension reactions Primer extension reactions
were carried out on 10 μg (wild-type) or 10 μg (ΔrpoB) of
total leaf RNA as described (Allison and Maliga, 1995

15   EMBO J. 15:2802-2809). The primers are listed below.
Underlined oligonucleotides were also used to generate
the capping constructs.

Gene	5' nucleotide position in plastid DNA	Sequence
accD	59758	<u>CCGAGC</u>TCTTATTTCCTATCAGACTAAGC
atpB	56736	CCCCAGAACCAGAAGTAGTAGGATTGA
atpI	15973	GTATTGATGGAACATGATAGAACAT
clpP#1	74479	GGGACTTTTGGAACACCAATAGGCAT
clpP#2	74947	<u>GGGAGC</u>TCCATGGGTTTGCCTTGG
ORF1901	31451	CTTCATGCATAAGGATACTAGATTACC
ORF2280	87419	<u>GGGAGC</u>TCTACATGAAGAACATAAGCC
rps2	16921	CCAATATCTTCTTGTCATTTCTCTC
rps16	6185	CATCGTTTCAAACGAAGTTTTACCAT

30   Sequence ladders were generated with the same primers
using the Sequenase II kit (USB).

Identification of primary transcripts by in vitro capping

35   Total leaf RNA (20 mg) from wild-type and ΔrpoB plants
was capped in the presence of [a-32P]GTP (Kennell and

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- 27 -

Pring, 1989 Mol Gen. Genet., **216**:16-24). Labeled RNAs
were detected by ribonuclease protection (Vera and
Sugiura, 1992, supra) using the RPAII kit (Ambion). To
prepare the protecting complementary RNA, the 16SrDNA
5   upstream region (nucleotides 102526-102761 of the ptDNA)
was PCR-amplified using the primers listed below. The 5'
primers were designed to add an XbaI restriction
site(underlined) upstream of the amplified fragment. The
3' primers were designed to add a KpnI site (underlined)
10   downstream of the amplified sequence. The amplified
product was cloned as an XbaI to KpnI fragment into XbaI-
and KpnI-restricted pBSKS+ vector (Stratagene). To
generate unlabeled RNA complementary to the 5' end of
RNAs, the resulting plasmid was linearized with XbaI, and
15   transcribed in a Megascript (Ambion) reaction with T3 RNA
polymerase. Markers (100, 200, 300, 400, and 500
nucleotides) were prepared with the RNA Century Markers
Template Set (Ambion), following the manufacturer's
protocol.

20

Gene	5' nucleotide position in plastid DNA	Sequence
accD	59758	CCGAGCTCTTATTTCCTATCAGACTAAGC
	59576	CCGGTACCATAGGAGAAGCCGCCC
atpB	56750	CCGAGCTCGTAGTAGGATTGATTCTCA
	57131(C)	CCGGTACCGGAGCCAATTAGATACAAA
atpI	15895	CCGAGCTCTGACTTGGAAACCCCC
	16277(C)	CCGAATTCTAGTATTCGCAATTTGT
clpP	74462	GGGAGCTCCAGGACTTCGGAAAGG
	74752(C)	GGGGTACCAATACGCAATGGGG
	74947	GGGAGCTCCATGGGTTTGCCTTGG
	75080(C)	GGGGTACCGCTAATTCATACAGAG
ORF1901	31424	GGGAGCTCCGACCACAACGACCG
	31724(C)	GGGGTACCCTTACATGCCTCATTTC
ORF2280	87419	GGGAGCTCTACATGAAGAACATAAGCC
	87154	GGGGTACCGTGCCTAAGGGCATATCGG

SUBSTITUTE SHEET (RULE 26)

- 28 -

DNA sequence analysis DNA sequence analysis was carried out utilizing the Wisconsin Sequence Analysis Package
5   (Genetics Computer Group, Inc.).

Results and Discussion

Based on the accumulation of mRNAs in wild-type and ΔrpoB leaves, the plastid genes may be divided into
10   three classes. The first class includes genes for which the mRNAs accumulate to high levels in wild-type leaves, and to very low levels in the leaves of ΔrpoB plants (Figure 6A). Genes which belong to this class are psaA (photosystem I gene), psbB and psbE (photosystem II
15   genes), petB (cytochrome b6/f complex gene), ndhA (respiratory chain NADH dehydrogenase homologue; Matsubayashi et al., 1987 Mol. Gen. Genet. 210:385:393) and rps14 (ribosomal protein gene). The second class includes plastid genes for which the mRNAs accumulate to
20   about equal levels in the wild-type and (rpoB leaves (Figure 6B). This class includes atpB (ATP synthase gene), ndhF (respiratory chain NADH dehydrogenase homologue gene; Matsubayashi et al., 1987, supra), rps16 (ribosomal protein gene) and ORF1901 (a gene with unknown
25   function; Wolfe et al., 1992, J. Mol. Biol. 223:95-104). The third class includes genes for which there is significantly more mRNA in the ΔrpoB leaves than in the leaves of wild-type plants (Figure 6C). Typical for this class are rpl33 and rpl18 (ribosomal protein genes),
30   accD (encoding a subunit of the acetyl-CoA carboxylase; Sasaki et al., 1993 Plant Physiol. 108:445-449) and ORF2280 (putative ATPase with unknown function; Wolfe 1994, Curr. Genet. 25:379-383). Two additional genes of this class, ndhB (respiratory chain NADH dehydrogenase
35   homologue; Matsubayashi et al., 1987, supra) and clpP (encoding the proteolytic subunit of the Clp ATP-dependent protease; Maurizi et al., 1990 J. Biol. Chem. 265:12546-12552; Gray et al., 1990 Plant Mol. Biol.

- 29 -

15:947-950) form a subgroup of this class which
demonstrate significant levels of mRNA in wild-type
leaves.

5

        The atpB and atpI ATP synthase genes have both
NEP and PEP promoters.
            The RNA gel blot analysis identified a number
    of genes and operons for which high transcript levels are
10 maintained in ΔrpoB leaves. To identify additional NEP
    promoters, the 5'-end of several transcripts has been
    mapped by primer extension analysis. 5' ends may be
    those of primary transcripts identifying a promoter, or
    generated by RNA processing. Since primary plastid
15 transcripts retain triphosphate groups at their 5' ends,
    specific [^{32}P]GMP transfer to these RNA molecules by the
    enzyme guanylyltransferase allowed accurate
    discrimination between primary transcripts and processed
    ends. For the tobacco atpB operon, transcript 5'- ends
20 have been identified by Orozco et al. (1990 Curr. Genet.
    17:65-71.) at nucleotide positions -611, -502, -488,
    -289 and -255 upstream of the translation initiation
    codon (Figure 7C). The 5' ends are numbered relative to
    the translation initiation codon (ATG) when the
25 nucleotide directly upstream of A is at position -1.
    Primer extension analysis identified each of these
    5'-ends in our wild-type plants (Figure 7A). In the ΔrpoB
    sample only the -289 RNA species was present, the 5' end
    of which was a substrate for guanylyltransferase (Figure
30 7B). Therefore, the -289 RNA is transcribed from a NEP
    promoter, PatpB-289. Interestingly, the -289 transcript
    is present in the wild-type leaves, although it is less
    abundant than in the ΔrpoB plants. The -255, -488 and
    -611 transcripts are absent in the ΔrpoB plants (Figure
35 7A). DNA fragments containing these promoters (but not
    PatpB-289) are recognized by the E. coli RNA polymerase
    (Orozco et al., 1990, supra), and are transcribed by PEP

in plastids.   The atpA operon includes the atpI,
-atpH-atpF-atpA genes (Figure 8C).   In wild-type tobacco
leaves,   mRNA 5' ends have been mapped to three regions
upstream of atpI,: the -209 region, with 5'ends mapping

5   to nucleotides -212, -209 and -207, and 5'-ends at
nucleotides -130 and -85.   In ΔrpoB leaves only the -207
transcript is detectable (Figure 8A).   This transcript
could be capped in the ΔrpoB RNA sample (Figure 8B),
therefore it is transcribed from a NEP promoter.   A

10   signal at this position was also obtained in the in vitro
capping reaction of wild-type RNA samples.   The -209 and
-212 transcripts may be due to the activity of an
overlapping PEP promoter, or formation of multiple
transcripts from the NEP promoter in wild-type plants.

15   The -130 transcript which is present only in wild-type
leaf RNA could also be capped (Figure 8A, 8B).   Since
there are sequences similar to -10/-35 elements at the
correct spacing upstream of this 5'-end, it is
transcribed by the PEP polymerase.

20

         **A clpP NEP promoter is highly expressed in
chloroplasts.**
         The clpP protease subunit gene also belongs to
the class which has both NEP and PEP promoters.   Primer

25   extension analysis in the wild-type plants identified RNA
5'-ends at nucleotide positions -53, -95, and -173, while
in ΔrpoB plants 5' ends map to the -53, -173, and -511
nucleotide positions (Figure 9A).   In vitro capping
reaction verified that each of these are primary

30   transcripts (Figure 9B).   Three of the transcripts derive
from NEP promoters.   The PclpP-53 promoter is highly
expressed in both wild-type-and ΔrpoB plants, thus
represents a distinct class of NEP promoters with a
potential for high-level expression in different tissue

35   types.   The PclpP-53 promoter is well conserved in
spinach (Westhoff, 1985 Mol. Gen. Genet. 201:115-123).
Additional clpP promoters for NEP are PclpP-173 and

PclpP-511. Since the PclpP-511 transcript accumulates only in ΔrpoB plants (Figure 9A) it is a candidate regulated NEP promoter. Note also, that the PclpP-511 is located within the psbB coding region, and it's

5   expression may be affected by the convergent psbB PEP promoter (Figure 9C).

The only PEP promoter directly upstream of clpP is PclpP-95. RNAs from this promoter accumulate only in wild-type leaves and PclpP-95 has upstream sequences

10   reminiscent of the -10-/-35 conserved elements (not shown).

The accD gene is transcribed exclusively from a NEP promoter.

15   For the lipid biosynthetic gene accD, mRNA accumulates to high levels only in ΔrpoB plants. A major transcript initiates at nucleotide position -129 (Figure 10A), which can be capped in vitro (Figure 10B). Therefore, this RNA is transcribed from a NEP promoter.

20   Since PaccD-129 does not have a significant activity in the photosynthetically active leaf mesophyll cells, it serves as a candidate for a regulated NEP promoter with a distinct tissue-specific expression pattern.

25   **NEP promoters share a loose consensus adjacent to the transcription initiation site.**

Sequences flanking the transcription initiation sites were aligned to identify conserved NEP promoter elements (Figure 11). Included in the sequence alignment

30   are nine promoters identified in this study and Prrn-62, the NEP promoter described in Allison et al. (1996, supra). Sequences for PORF2280-1577, and PORF1901-41 for which the 5' ends were shown to be primary transcripts by capping in vitro are also included (data not shown).

35   Both of these promoters are active in ΔrpoB leaves but not in the leaves of wild-type plants. Also included in the sequence alignment are tentative NEP promoters for

- 32 -

rps2 and rps16 , for which there is more mRNA in ΔrpoB
leaves. The 5' ends of these transcripts were mapped by
primer extension analysis. The in vitro capping assay
failed due to low abundance of the mRNAs (data not

5   shown). Multiple sequence alignment of the regions
immediately flanking the NEP 5' ends identified a loose
10 nucleotide consensus around the transcription
initiation site (Figure 11). Conservation of additional
nucleotides upstream and downstream is also apparent..

10   Striking is the lack of sequence conservation between the
PclpP-53 and other NEP promoters which is the only NEP
promoter highly active in chloroplasts. Given the lack of
sequence similarity, this sequence was not included in
the alignment. Sequences around the PclpP-53

15   transcription initiation site are shown separately at the
bottom of Figure 11.

        NEP and PEP polymerases, through recognition of
distinct promoters, provide a mechanism for selective
transcription of plastid genes (Figure 12). The data

20   provided herein demonstrate that some genes have only PEP
promoters or NEP promoters while others have both PEP and
NEP regulatory sequences.

EXAMPLE III

NEP Promoters for the Expression of Selectable Marker
25   **Genes**

        For versatility and universal applications,
expression of selectable marker genes for plastid
transformation is desirable in all tissue types at a high
level. Selectable marker genes in the currently utilized

30   plastid transformation vectors are expressed from PEP
promoters recognized by the plastid encoded RNA
polymerase. The PEP polymerase transcribes
photosynthetic genes and some of the housekeeping genes,
therefore appears to be the dominant RNA polymerase in

35   photosynthetically active leaf tissues. Efficient plastid
transformation has been achieved in tobacco based on
chloroplasts transformation in leaf cells. However,

plant regeneration is not feasible, or is not practical
from the leaves of most agronomically important cereal
crops, including maize, rice, wheat and in cotton. In
these crops, transgenic plants are typically obtained by
5   transforming embryogenic tissue culture cells or seedling
tissue. Given that these tissues are non-photosynthetic,
expression of marker genes by NEP promoters which are
active in non-green tissues appears to be particularly
advantageous, and will facilitate transformation of
10   plastids in all non-photosynthetic tissue types.
        A particularly suitable promoter to drive the
expression of marker genes is the PclpP-53 promoter.
This promoter is highly expressed in the proplastids of
ΔrpoB plants, therefore it may also be highly expressed
15   in the proplastids of embryogenic cell cultures which
yield transgenic cereal plants. Marker genes expressed
from these promoters will also be suitable to select
plastid transformants in bombarded leaf cultures, since
this promoter was found to be active in chloroplasts.
20   Marker genes expressed from promoters such as the
PclpP-53 promoter will have wide application to obtain
transformed plastids.
        The selectable marker genes will be constructed
using the principles outlined in U.S. Patent No.
25   5,451,513 and pending U.S. Application Serial No.
08/189,256, the subject matter of which is incorporated
by reference herein. A transforming DNA construct is
illustrated in Figure 13. More specifically, the
PclpP-53 promoter will be cloned upstream of a DNA
30   segment encoding a plastid-selectable marker. Signals for
translation will be provided by incorporating suitable
DNA sequences between the promoter fragment and the
selectable marker coding region. 3' untranslated
segments of a plastid gene to provide signals for
35   transcription termination and to stabilize the chimeric
mRNA will be cloned downstream of the selectable marker.
Utilization of the 3'untranslated region of genes

expressed from NEP promoters is preferred since the requirements for transcription termination for the NEP and PEP polymerases may be different.

PclpP-53 is a particularly strong NEP promoter.
5 However, plants with transformed plastids may be obtained with weak promoters as well. There are several examples for such weak NEP promoters in the preceding examples, for example PclpP-173.

10

Expression of Tissue Specific Plastid Transgenes Driven
15 by NEP Promoters

Tissue specific expression of plastid transgenes is desirable in many applications. Tissue specific expression of a protein that makes the plant
20 tissue repellent or toxic for root nematodes may be desirable in roots. However, expression of the same protein in the leaves would drain the plants resources and may effect utilization of the aerial plant parts. Since most often expressed in non-green tissues, the NEP
25 promoters described in this application, and the promoters expressed from the NEP polymerase in general, are a rich source of tissue-specific promoters for transgene expression.

Several of the NEP promoters, for example
30 PclpP-511, are highly expressed in proplastids of ΔrpoB plants. Proplastids are present in the edible part of cauliflower. Therefore, high level expression of foreign genes in cauliflower is anticipated from this promoter in the edible parts of the plant.

35 The plastid gene accD encodes a subunit of the prokaryotic acetyl-CoA carboxylase, an enzyme involved in lipid biosynthesis. Interestingly, in wild-type leaves the level of accD mRNA is low while it is high in the proplastids of ΔrpoB plants. This observation suggests
40 that PaccD-129 is active in non-green plastids of tissues

actively involved in lipid biosynthesis, such as the
plastids of developing seed which is rich in oil.

What is claimed is:

1.   A DNA construct for stably transforming plastids of multicellular plants, which comprises a transforming DNA having a targeting segment which effects insertion of said transforming DNA into said plastid genome by homologous recombination, a selectable marker gene conferring a selectable phenotype to plant cells containing said transformed plastids and a cloning site for insertion of an additional expressible DNA encoding a foreign gene of interest, wherein the improvement comprises a 5' promoter element which is recognized and transcribed by a nuclear encoded plastid RNA polymerase.

2.   The DNA construct of claim 1, wherein said construct is incorporated into a vector suitable for transformation of plastids.

3.   A DNA construct according to claim 1, wherein said 5' promoter element is recognized and transcribed by a plastid encoded plastid RNA polymerase.

4.   The DNA construct according to claim 1 wherein said promoter element is selected from promoter elements of plastid genes selected from the group consisting of Prrn-62, PORF2280-1577, PatpB-289, PORF1901-41, Prbs2-152, Prps16-107, PatpI-207, Pclp-511, Pclp-173, Pclp-53 and PaccD-129.

5.   A DNA construct according to claim 2, wherein said promoter element is Pclp-95.

6.   A DNA construct for stably transforming the plastids of a plant cell and for expression of at least one additional gene product therein, comprising:
         a)   a targeting segment comprising a DNA sequence substantially homologous to a predetermined

plastid genomic sequence with a plastid to be
transformed, said targeting segment enabling homologous
recombination with said pre-determined plastid genomic
sequence; and

5   b) a selectable marker gene disposed within
said targeting segment, said selectable marker gene
conferring a non-lethal selectable phenotype to cells
containing plastids with said DNA construct; and

           c)   an additional DNA segment comprising a
10   transcription unit of a gene encoding a protein or a
precursor thereof; and

           c)   a promoter sequence which is operably
linked to said transcription unit, said promoter sequence
being recognized by a nuclear encoded polymerase, said
15   gene encoding said protein being regulated by said
promoter.

           7.   A DNA construct according to claim 6,
wherein said transcription unit encodes a selectable
20   marker gene.

           8.   A DNA construct according to claim 7
wherein said selectable marker gene is regulated by a
promoter transcribed by a nuclear encoded plastid
25   polymerase.

           9.   A DNA construct according to claim 6
wherein said transcription unit encodes a reporter gene.

30   10.   A DNA construct according to claim 6,
wherein said construct is incorporated into a vector
suitable for transformation of plastids.

           11.   A multicellular plant stably transformed
35   with the DNA construct of claim 1.

           12.   A multicellular plant stably transformed

with the DNA construct of claim 2.

13.   A method for obtaining a plant cell or a
multicellular plant, the plastids of which cell have been
stably transformed by at least one foreign gene of
interest, which comprises administering to a plant cell a
DNA construct comprising:
        a)   a targeting segment comprising a DNA
sequence substantially homologous to a predetermined
plastid genomic sequence with a plastid to be
transformed, said targeting segment enabling homologous
recombination with said pre-determined plastid genomic
sequence; and
        b) a selectable marker gene disposed within
said targeting segment, said selectable marker gene
conferring a selectable plastid phenotype to cells
containing plastids with said DNA construct; and
        c)   and a foreign gene of interest, said gene
being regulated by a promoter recognized by a nuclear
encoded plastid RNA polymerase;
        d)   selecting for cells which express said
phenotype; and
        e)   regenerating a plant from said cell
containing stably transformed plastids.
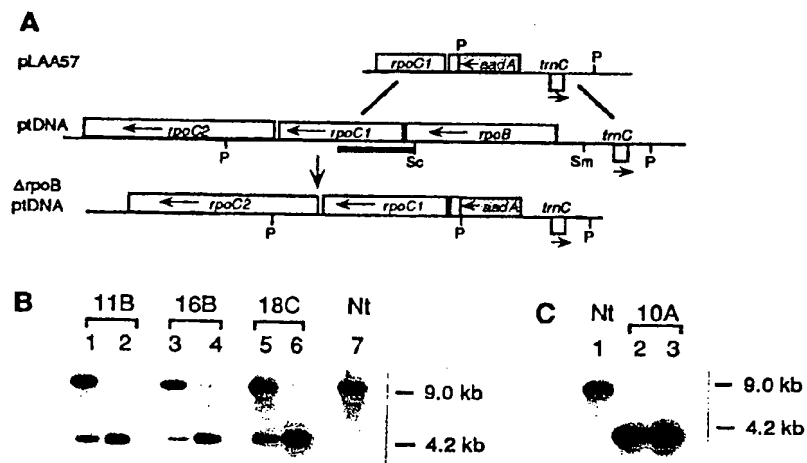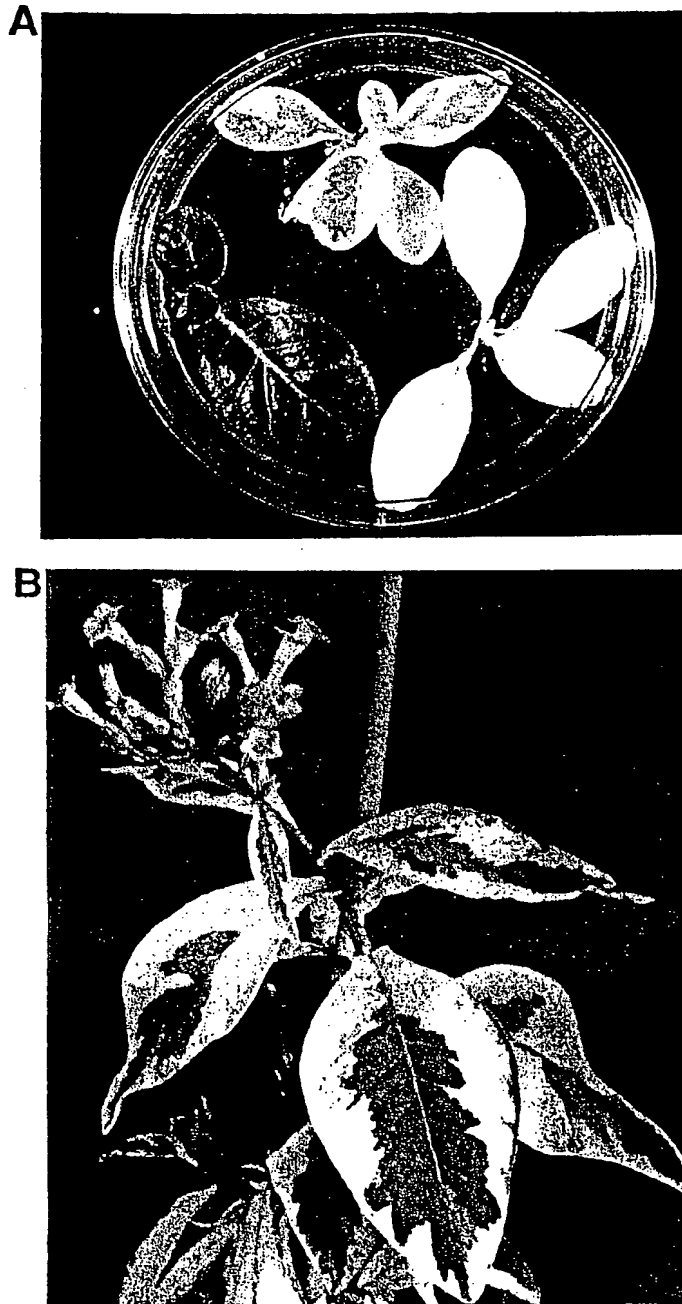
1/13

Figure 1

Figure 2

Fig. 3A

Fig. 3B

Figure 3

4/13

Figure 4

A
rbcL
1 2

16S rRNA
3 4

B
16S rRNA
1 2 3

C
-201 ATTTGCTCCCCCGCCGTCGTTCAATGAGAATGGATAAGAGGCTCGTGGGA

-114,-113 (P1)

-151 TTGACG TGAGGGGGGCAGGGATGGC TATATT TCTGGGAGCGAACTCCGGGC

-62 (P2)

-101 GAATATGAAGCGCATGGATACAAGTTATGCCTTGGAATGAAAGACAATTC

-51 CGAATCCGCTTTGTCTACGAACAAGGAAGCTATAAGTAATGCAACTATGAA

Figure 5

Figure 6

Figure 7

8/13


Figure 8

Figure 9

A

A C G T   T57 wt

B

T57   MW   wt
1   2   3   4   5

● 129

C

rbcL   accD

-129

Figure 10

Prrn-62
PORF2280-1577
PatpB-289
Prps2-152
Prps16-107
PORF1901-41
Patpl-207
PclpP-511
PclpP-173
PaccD-129

Prrn-62
PORF2280-1577
PatpB-289
Prps2-152
Prps16-107
PORF1901-41
Patpl-207
PclpP-511
PclpP-173
PaccD-129

Consensus

PclpP-53

FIGURE 11

NEP polymerase   PEP polymerase

FIGURE 12

13/13

PclpP-53   Selectable marker   TclpP

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Translational
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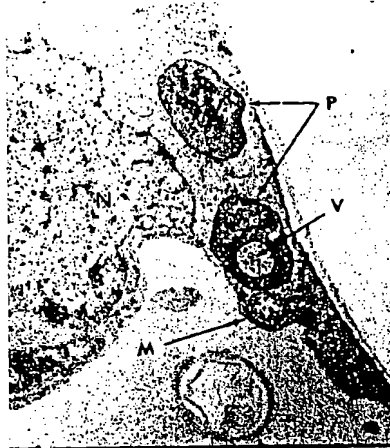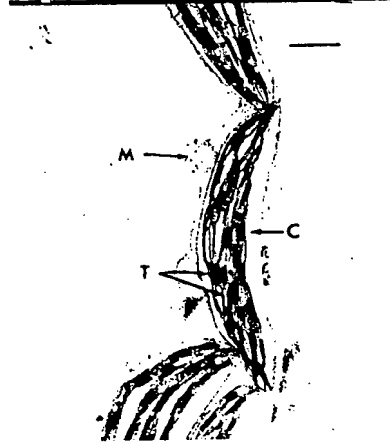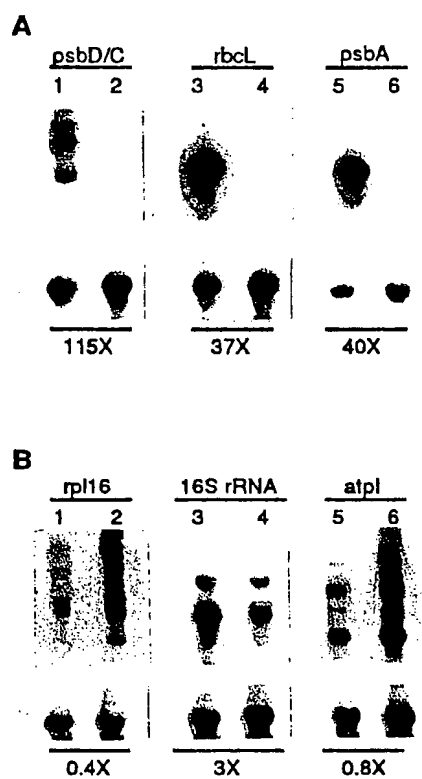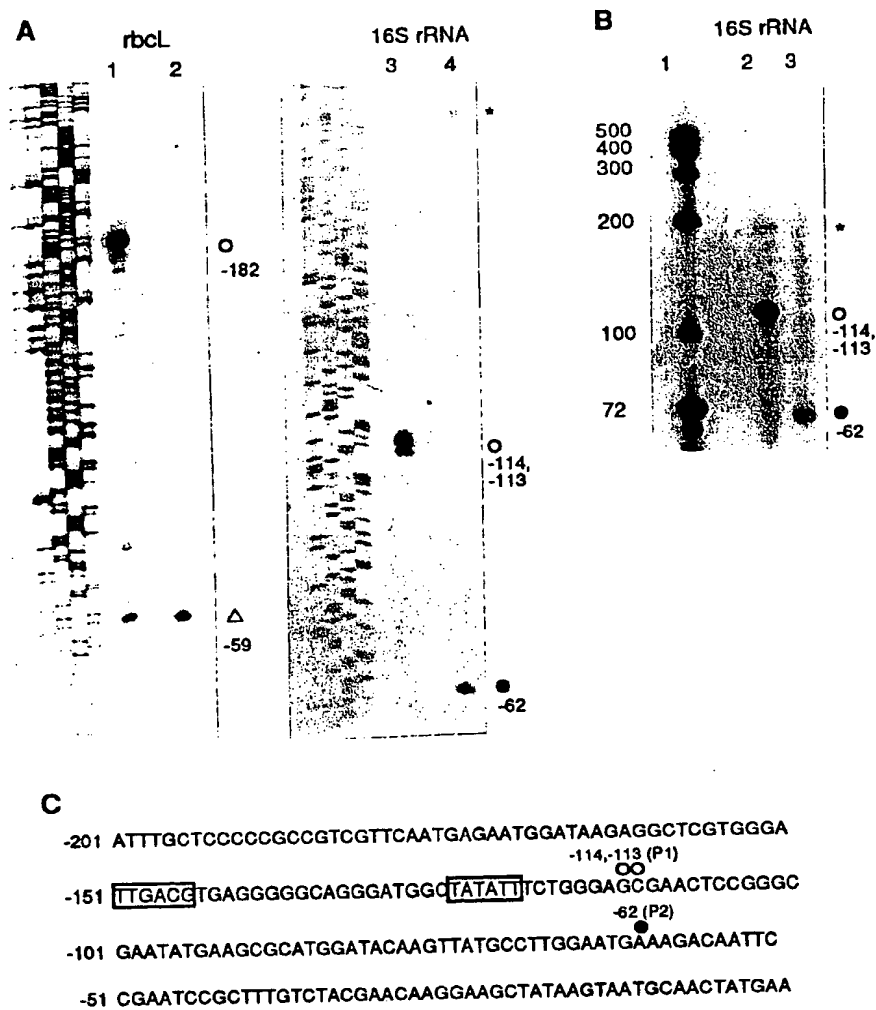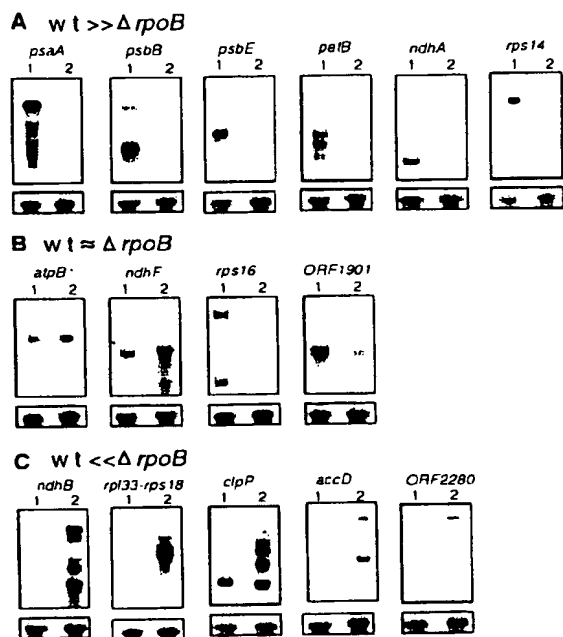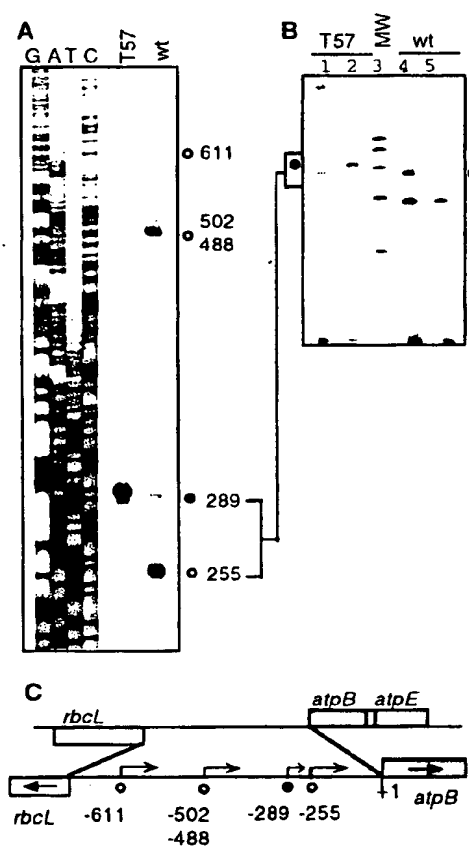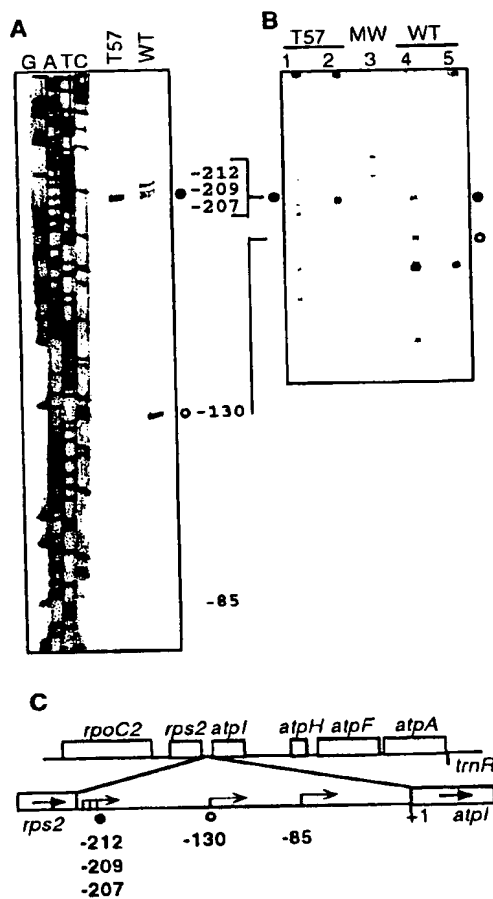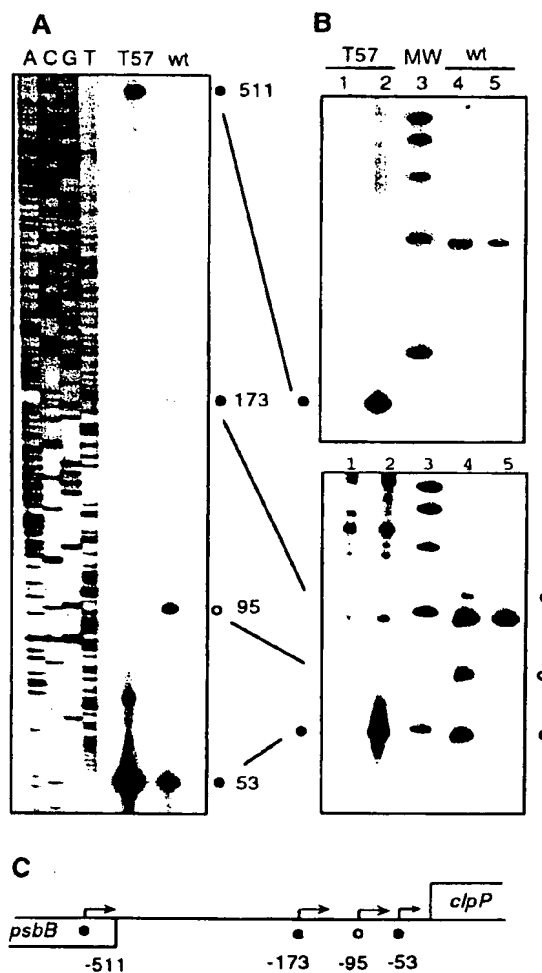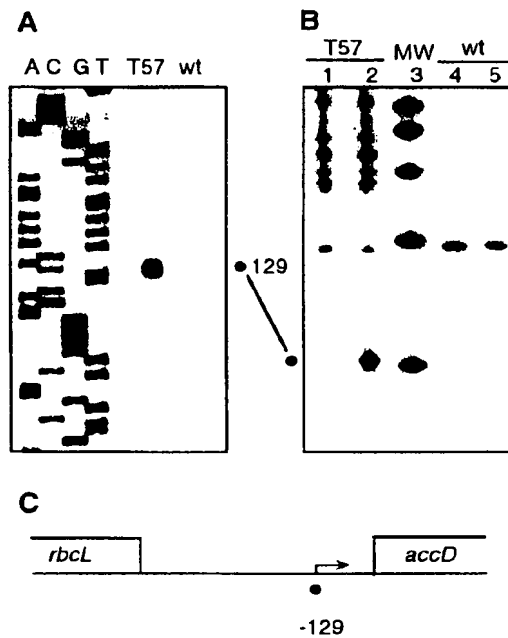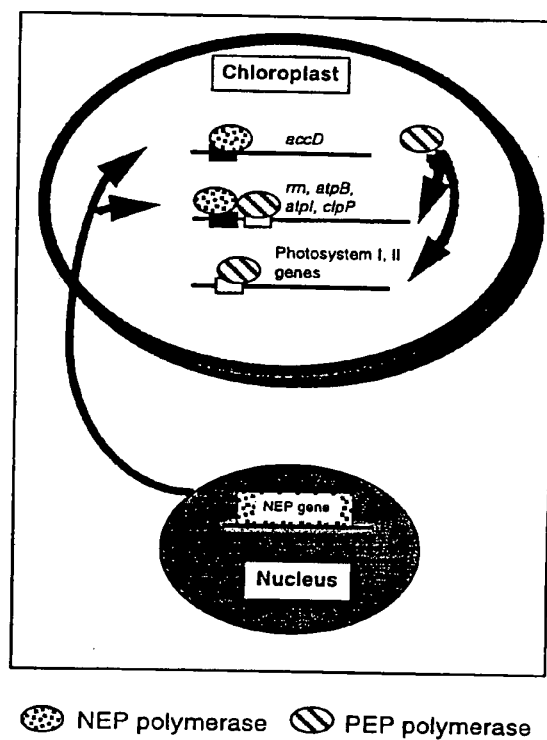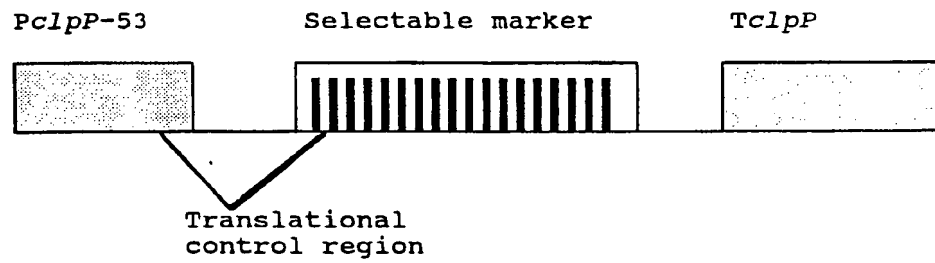
FIGURE 13

INTERNATIONAL SEARCH REPORT

A. **CLASSIFICATION OF SUBJECT MATTER**	

IPC(6)   :Please See Extra Sheet.
US CL   : 435/172.1, 172.3, 240.4, 320.1; 536/23.1, 24.1; 800/205

According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/172.1, 172.3, 240.4, 320.1; 536/23.1, 24.1; 800/205

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	STAUB et al. Long Regions of Homologous DNA Are Incorporated into the Tobacco Plastid Genome by Transformation. The Plant Cell. January 1992, Vol. 4, pages 39-45, especially pages 39 and 42-43.	1-13
Y	MULLET, JOHN E. Dynamic Regulation of Chloroplast Transcription. Plant Physiol. 1993, Vol. 103, pages 309-313, especially pages 309-312.	1-13
Y	TONKYN et al. Differential expression of the partially duplicated chloroplast S10 ribosomal protein operon. Mol. Gen. Genet. 1993, Vol. 241, pages 141-152, especially pages 141-142 and 148-152.	1-13

[X] Further documents are listed in the continuation of Box C.	[ ] See patent family annex.

*   Special categories of cited documents:

"A"   document defining the general state of the art which is not considered to be of particular relevance

"E"   earlier document published on or after the international filing date

"L"   document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P"   document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
22 OCTOBER 1996	04 NOV 1996

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INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SVAB et al. High-frequency plastid transformation in tobacco by selection for a chimeric aadA gene. Proc. Natl. Acad. Sci. USA. February 1993, Vol. 90, pages 913-917, especially pages 914-916.	1-13
Y,P	US 5,451,513 A (MALIGA et al) 19 September 1995, columns 1-5.	1-13

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12N 15/00, 15/09, 15/11, 15/29, 15/63, 15/65, 15/82, 15/90; AO1H 1/00, 1/04